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Compositional mixed modelling of methane emissions and ruminal volatile fatty acids from individual cattle and multiple experiments[1]

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ABSTRACT: The aim of the study was to investigate the association of methane (CH_4) yields (g/kg DMI) with rumen VFA molar proportions and animal and diet-related covariates from individual animals and multiple experiments. The dataset available consisted of 284 measurements of CH_4 yields for beef cattle from 6 experiments measured in indirect respiration chambers. A compositional modelling approach was employed where VFA measurements were considered as a whole, instead of in isolation, emphasizing their multivariate relative scale. The analysis revealed expected close groupings of acetate and butyrate; propionate and valerate; iso-butyrate and iso-valerate. Linear mixed models were then fitted to examine relationships between CH_4 yield and VFA, represented by meaningful log-contrasts of components called compositional balances, while accounting for other animal and diet-related covariates and random variability between experiments. A compositional balance representing (acetate · butyrate)/propionate best explained the contribution of VFA to variation in CH_4 yield. The covariates DMI, forage:concentrate proportion (expressed as a categorical variable diet type: high concentrate, mixed forage:concentrate or high forage), and diet ME were also statistically significant. These results provided new insights into the relative inter-relationships amongst VFA measurements and also between VFA and CH_4 yield. In conclusion, VFA molar proportions as represented by compositional balances were a significant contributor to explaining variation in CH_4 yields from individual cattle.

Key words: methane production, volatile fatty acids, compositional data, mixed models.

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INTRODUCTION

Methane (CH_4), derived almost entirely (90%) from enteric fermentation, is a major contributor to greenhouse gas emissions from the livestock sector, and cattle are responsible for most (77%) of CH_4 emissions (Gerber et al., 2013). While modifying the diet of cattle is the most effective short-term method for mitigating CH_4 emissions, longer-term the wide variation in CH_4 yield (g CH_4/kg DMI) between individual animals (up to 2-fold when fed the same diet, Rooke et al., 2014) must be exploited. A limitation to this is the relatively slow output achievable using indirect respiration chambers to measure CH_4 yield and the cost of such measurements. The amount of CH_4 produced from a specific diet by rumen archaea depends largely upon the amount of hydrogen (H_2) produced as an end-product of fermentation of feed carbohydrates by other organisms in the rumen microbiome. There are well established stoichiometric relationships between the pattern of VFA and H_2 produced by rumen fermentation and resulting CH_4 formation (Wolin, 1960; Murphy et al., 1982; Alemu et al., 2011), but these stoichiometric relationships have usually been modelled using data at the diet level. In the current study, we use CH_4 and VFA measurements for individual animals from 6 experiments to address the hypothesis that VFA pattern in an individual animal could be used as an explanatory variable in accounting for variation in CH_4 yield in addition to other diet and animal characteristics. A key methodological novelty is that we consider VFA (expressed as molar proportions) as a composition of intrinsically co-dependent amounts carrying only relative information. This was embedded into a linear mixed modelling framework to account for the variability originating from the multiple study structure of the data.

MATERIALS AND METHODS

The experiments which were included in the database were conducted at Scotland's Rural College (SRUC) Beef and Sheep Research Centre in Edinburgh. Each individual experimental protocol was approved by SRUC's Animal Welfare and Ethical Review Body, the Animal Experiments Committee and was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act, 1986.

Description of the Data

The data analyzed here were obtained from 6 experiments carried out at SRUC between 2011 and 2014. The cattle used were either steers (Rooke et al., 2014, Troy et al., 2015, 2016, Duthie et al., 2017; exp. 1–4) or beef cows (Duthie et al., 2015; exp. 5 (unpublished) and of varying breed types (Aberdeen Angus x Limousin; Limousin x Aberdeen Angus; Luing; Charolais cross bred; exp. 6, Aberdeen Angus x Limousin and Limousin x Aberdeen Angus). Diets fed were of 3 types which were used as a categorical variable, diet type, in analysis. High concentrate diets (Concentrate; < 100 g forage/kg DM) were based on ground barley and barley straw with either rapeseed meal or distillers dark grains (similar to distillers grains with solubles but low in sulfur). Mixed forage:concentrate diets (Mixed; 400 – 600 g forage/kg DM) were based on grass silage, whole crop barley silage, barley, and either rapeseed meal or distillers dark grains. High forage diets (Forage; > 700 g forage/kg DM) consisted of barley straw and either grass silage or brewers' grains (Duthie et al., 2015) or a mixture of grass silage (696 g/kg DM) and whole crop barley silage (293 g/kg DM) in exp. 5. All diets were offered ad libitum as total mixed rations (fed once daily) and had been fed for at least 3 weeks before measurements of CH_4 yield. Methane output was measured (48 h) using indirect respiration chambers. As it was not possible to take rumen samples whilst animals were in the chambers, a single sample of rumen fluid for VFA analysis was taken by stomach tube within 2 h of cattle leaving the chambers

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(approximately 25 h after feed bins were last refilled). Full details of methodology can be found in Rooke et al. (2014); Duthie et al. (2015) and Troy et al. (2015). The constituents of the diets are summarized in Table 1 and mean cattle BW, DMI, and CH_4 production in Table 2. It should be noted that only animals which had complete records of DMI, CH_4, and VFA were used in the current analysis; thus, there may be small discrepancies from the original references.

TABLE 1 (DIET CONSTITUENTS)

TABLE 2 (SUMMARY OF VARIABLES)

VFA Measurements as Compositional Data

Data accounting for relative parts of a whole are known as compositional data (see e.g. Aitchison, 1986; Pawlowsky-Glahn et al., 2015 for discussion, formal properties and principles). This is the case for chemical mixtures such as VFA composition when expressed as portions of the whole in either weight or volume units. Thus, the components of the VFA mixture are intrinsically co-dependent positive amounts carrying only relative information. Changes in one or several components affect the remaining ones and, consequently, an equivalent symmetric overall change should be measured on the latter as well. The relative and symmetric scale is commonly recognized in practice by re-expressing the data in proportions adding up to 1 or similar units like mmol/mol used here. It is important to note that the data do not necessarily have to add up to the same constant total. Given a *D*-part composition $\mathbf{x} = [x_1, \dots, x_D]$, the statistical analysis focuses on the log-ratios $\ln(x_i/x_j)$ between components. Using this type of transformation, the results do not depend on the units of measurement and the relative scale of the data is considered. It does not matter either whether the original full mixture or a subset (subcomposition) of components, which may or may not add to total VFA, are used as here.

Basic Compositional Statistics for the VFA Data Set

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Following Pawlowsky-Glahn and Egozcue (2002), the composition best representing the center of a data set $\mathbf{X} = [x_{ij}]_{n\times D}$ consisting of n compositional samples $\mathbf{x}_i$ of D components is given by the so-called compositional geometric mean (**CGM**), or compositional center, as $\mathrm{CGM}(\mathbf{X}) = C(g_1, \dots, g_D)$, where $g_j = \left(\prod_{i=1}^{n} x_{ij}\right)^{1/n}$, for $j = 1, \dots, D$, is the geometric mean of the the jth column of $\mathbf{X}$. The closure operator C normalizes the resulting vector of geometric means to be expressed in the chosen scale. For example, if working with proportions, C would mean multiplying each component by $1/\sum_{j=1}^{D} g_j$ so that the total sum of each composition is 1. Moreover, instead of using ordinary correlations, the relative variability structure of $\mathbf{X}$ is given by the matrix of log-ratio variances $\mathbf{T} = [\tau_{ij}]_{D\times D}$, where $\tau_{ij} = \mathrm{var}\left(\ln\left(x_i/x_j\right)\right)$, for $i, j = 1, \dots, D$, with var referring to the ordinary variance measure. Relationships between components are then understood in terms of proportionality. A log-ratio variance which is close to 0 indicates that 2 components x_i and x_j are nearly proportional (highly co-dependent); that is, their log-ratio is nearly constant. A measure of global dispersion is provided by the total variance $\mathrm{totvar}(\mathbf{X}) = 1/2D \sum_{i,j=1}^{D} \tau_{ij}$. Estimators of these measures from data are obtained by the standard maximum likelihood procedure.

Log-ratio Coordinate Representation

Recent advances in the area (Egozcue et al., 2003) allow the definition of isometric (metric-preserving) mappings between the original composition $\mathbf{x}$ and log-ratio coordinates living in the ordinary real space, which facilitates the use of standard statistical methods. These isometric log-ratios (**ilr**) can be constructed in infinitely many different ways, although they are essentially orthogonal rotations of one to another. This means that results from any set of ilr coordinates transform back into the same results in terms of the original composition. Note that compositions of size D correspond to vectors of isometric log-ratio coordinates of size $D - 1$ (the actual degrees of freedom of the composition). A procedure known as sequential binary partition (**SBP**; Egozcue and Pawlowsky-Glahn, 2005) allows the

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construction of tailored ilr coordinates, usually called compositional balances (b_i, where $i = 1, \ldots, D-1$), representing log-contrasts between subsets of components of $\mathbf{x}$. This is of great practical relevance because balances can be defined according to insights from exploratory data analysis (see Model A1 below) or using biological knowledge.

Building Balances for the VFA Composition

Balances are obtained using SBP by successive splits of the components of the VFA composition $\mathbf{x}$ into 2 mutually exclusive groups until only groups of 1 component are left (see left-hand side of Table 4). These two groups are coded by the signs + and – respectively. The collection of $D-1$ balances b_i, for $i = 1, \ldots, D-1$, is obtained as

$$b_i = \sqrt{\frac{r_i s_i}{r_i + s_i}} \ln \frac{\left(\prod_{k=1}^{r_i} x_{ik}^{+}\right)^{1/r_i}}{\left(\prod_{k=1}^{s_i} x_{ik}^{-}\right)^{1/s_i}}, \qquad [1]$$

where x_{ik}^{+} and x_{ik}^{-} refer to the subsets of r_i and s_i components going, respectively, into the + (numerator) and – (denominator) groups. The $D-1$ balances fully represent the information in the composition $\mathbf{x}$ and, as previously said, they are appropriate to be used in standard statistical modelling. Note that the log-ratio term in Eq. [1] is computed as the ratio between the geometric means of the corresponding + and – components. It is multiplied by a normalizing constant to give the b_i that allow balances to be compared and confers on them desirable geometric properties. A balance then measures the relative importance, in geometric mean, of one group against the other by means of a log-contrast between them. Table 4 (left) depicts two alternative but equivalent balance representations of the VFA composition used in this work to produce a linear mixed model for CH_4 emissions. For example, the balance b_1 in Model A1 was computed as

$$b_1 = \sqrt{\frac{8}{6}} \ln \frac{(\text{iso-butyrate} \cdot \text{iso-valerate} \cdot \text{butyrate} \cdot \text{acetate})^{1/4}}{(\text{valerate} \cdot \text{propionate})^{1/2}}. \qquad [2]$$

In Model A2, the SBP is modified to isolate a balance of particular biological interest (balance b_2 in Model A2) so that its significance can be statistically tested. Note that, regardless of the balance representation chosen, it holds that $\mathrm{totvar}(\mathbf{X}) = \sum_{i=1}^{D-1} \mathrm{var}(b_i)$ for a given compositional data set **X**. Thus, the balances can be ranked according to their contribution to explaining the total variability within the data set.

A Compositional Linear Mixed Model for CH_4 Emissions

A linear mixed model (**LMM**) approach was adopted to integrate quantitative findings from the 6 different studies. The linear association between CH_4 emissions expressed as g/d or g/kg DMI (see Table 2) was moderately high and positive (Pearson's $r = 0.75$). Accordingly, similar estimates of the effects of the given explanatory variables would be obtained. For the purpose of this work we used CH_4 yields expressed in g/kg DMI as the response variable. Methane yield was log transformed to more closely satisfy the normality assumption of the model residuals. Formally, the response vector $\mathbf{y}_i$ from the ith experiment was modelled as

$$\begin{gathered} \mathbf{y}_i = \mathbf{B}_i \cdot \beta_1 + \mathbf{C}_i \cdot \beta_2 + \mathbf{Z}_i \cdot f_i + \varepsilon_i, \quad i = 1, \ldots, 6, \\ f_i \sim N(0, \sigma_f^2), \\ \varepsilon_i \sim N(\mathbf{0}, \sigma^2 \mathbf{I}), \end{gathered} \qquad [3]$$

where β_1 and β_2 were the coefficients of the fixed effects associated with, respectively, (a) the VFA balances ($\mathbf{B}_i$ matrix) as obtained from Eq. [1] given a SBP, and (b) the other explanatory covariates ($\mathbf{C}_i$ matrix). The BW and DMI summarized the information about animal covariates in the model. For diet-related covariates, diet type (Concentrate, Mixed or Forage) was included as an explanatory factor representing the diet contents (forage, starch, and neutral detergent fiber) across experiments, along with metabolizable energy (ME). All the covariates were log transformed to be introduced in the model. The term $\mathbf{Z}_i \cdot f_i$ was the experiment random effects term, with f_i assumed to be normally distributed with mean 0 and variance σ_f^2. The within-group random errors ε_i were assumed to be normally distributed

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with means 0 and variances σ^2. Random variability of model intercepts between experiments was assumed, which implied that the design matrix of the random effects $\mathbf{Z}_i$ equaled a unit vector. The random effects f_i and the random errors ε_i were assumed to be independent for different experiments and independent of each other for the same experiment.

Model fitting was conducted by restricted maximum likelihood estimation. The marginal statistical significance of the fixed effect coefficients was assessed by conditional *t*-tests (Pinheiro and Bates, 2000). Conditional *F*-tests were applied to jointly test for the significance of the VFA balance coefficients and, hence, of the VFA composition. Statistical significance was concluded when associated *P*-values were < 0.05. An approximate model goodness-of-fit measure for mixed models was provided by using the marginal and conditional R^2 coefficients for mixed models (R_m^2, % variance explained by fixed terms; and R_c^2, % variance explained by both fixed and random terms) proposed in Nakagawa and Schielzeth (2013). Note however that, due to the complications added by the random effects structure in mixed models, these are pseudo-R^2 coefficients. Hence, interpretation and comparison of model explained variances based on these coefficients must be conducted with extreme caution. Comparison of models with nested fixed effect structures and the same random effect structure was conducted using the Akaike information criterion (**AIC**) and likelihood ratio test (**LRT**) as obtained from maximum likelihood estimation of the models. Statistical analyses, including compositional analyses, and modelling were conducted in the R system for statistical computing v3.2 (R Core Team, 2016).

RESULTS

Exploratory Analysis of the VFA Composition

Table 3 shows compositional summary statistics for the VFA composition across all the experiments (other ordinary statistics for individual diets are supplied in Appendix 1).

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The overall CGM reveals that, as expected, acetate was the most abundant VFA (mean, 656 mmol/mol), whereas iso-butyrate, iso-valerate, and valerate were all present at < 20 mmol/mol. The results per diet type illustrate the differences in mean VFA profiles between them. The variation matrix indicates that acetate and butyrate held the strongest proportionality association ($\tau = 0.10$). Contrarily, propionate and butyrate or iso-valerate were the least proportionally associated components ($\tau = 0.29$).

TABLE 3 (VFA SUMMARY)

The relative variation structure of the data and the relationships between samples (points) and VFA components (rays) were approximately represented in Fig. 1 using a compositional biplot (Aitchison and Greenacre, 2002) explaining 68% of the data variability. The lengths of the links between arrowheads approximate the log-ratio variances (Table 3) of the corresponding components. Thus, propionate showed in general the greatest log-ratio variances (lowest proportionality) with all the others, particularly with iso-valerate and butyrate (relationship highlighted using dashed lines). The samples were distinguished by diet type. Concentrate diet type mostly associated with greater relative amounts of valerate and propionate, Forage diet type mostly linked to acetate and the Mixed diet type intermediate between Concentrate and Forage diet types but also linked to greater relative amounts of butyrate and iso-valerate.

FIGURE 1 (BIPLOT)

The variation matrix in Table 3 was used to arrange the VFA components into homogenous groups by the Ward's clustering method (Ward, 1963). This allowed for a hierarchical representation of the structure of proportionality relationships between VFA components in a dendrogram (Fig. 2).

FIGURE 2 (DENDROGRAM)

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Three groups of VFA components can be clearly distinguished: propionate and valerate, acetate and butyrate, and iso-butyrate and iso-valerate. This configuration is coherent with the biplot analysis above and can be used to define a set of compositional balances between VFA components based only on the proportionality structure inferred from the data. In particular, the balance contrasting propionate and valerate against the remaining VFA components (balance b_1 in Eq. [2]), corresponding to the top split in Fig. 2, explained most of the total variability (totvar) in the data set (34.42%) and was used as starting point to generate a collection of balances through SBP (Table 4, SBP for Model A1). Note that the subsequent balances b_2, b_3, b_4, and b_5 corresponded to log-contrasts between the components located at each of the two branches of the nodes of the dendrogram as indicated in Fig 2.

Associations between CH_4 Emissions, VFA Composition, and Diet and Animal Covariates

Model A1.

Table 4 (top, Model A1) shows parameter estimates and statistical significances from the model for CH_4 yield based on VFA balances according to the relative variation structure of the data as detailed above. All the balances in Model A1 but b_5 ($P = 0.832$) and b_4 ($P = 0.338$) were statistically significant. A joint test for the coefficients of the balances confirmed a statistically significant effect of the VFA composition as a whole ($F = 9.931$; $P < 0.001$). The Ln BW was the only covariate not statistically significant ($P = 0.129$). The marginal and conditional R^2 coefficients (64.65% and 68.60% respectively) reflected an acceptable model fit. Ordinary checks for normality and homogeneity of variances of the model residuals were satisfactory (not shown).

TABLE 4 (CLMM results)

Model A2.

The fact that some balances were not statistically significant in Model A1 raised the question of whether some VFA components were not relevant to explain CH_4 yield. Some further exploratory analysis suggested that the SBP used for Model A1 could be refined by using a balance representing the main VFA responsible for H_2 production (acetate and butyrate) and consumption (propionate) during carbohydrate fermentation. This was achieved by defining the SBP shown in Table 4 (bottom) for Model A2, with a new balance b_2 given by

$$b_2 = \sqrt{\frac{2}{3}} \ln \frac{(\text{acetate} \cdot \text{butyrate})^{1/2}}{\text{propionate}}. \quad [4]$$

Under this arrangement, which is simply a re-parametrization of Model A1, only the new balance b_2 was statistically significant. This result clearly picks out the log-contrast acetate-butyrate versus propionate (Eq. [4]) as the main driver of the relationship between CH_4 yield and VFA composition. It is important to stress at this point that Model A2 is entirely equivalent to Model A1, except for the use of a different set of ilr coordinates which allowed to test for the significance of a relationship of biological interest and enhanced interpretability. Overall measures, R^2 coefficients, AIC, model intercepts, estimates for the remaining covariates and random effects estimates were all identical. The technical reason for this is that different ilr transformations are orthogonal rotations of each other, and those estimates are invariant under such rotations. The AIC measure was used to rank the explanatory variables according to their relative importance by the sum of Akaike weights (Burnham and Anderson, 2002) over all possible models from Model A2 in which the variable was included. Note that all the statistically significant variables (diet type, Ln DMI, Ln ME, and b_2; see Table 4) were given the same Akaike weight of 1 (values range between 0 and 1) and, hence, they were all considered of analogous importance in the model.

Model B.

Instead of considering the entire VFA composition, we fitted here a simplified LMM for CH_4 yield based only on balance b_2 from Model A2 (Eq. [4]; summary statistics in

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Appendix 1) and the statistically significant covariates (see parameter estimates in Table 5). A LRT to compare Model A2 with Model B provided a statistically non-significant result (LR statistic = 6.36; $P = 0.273$) and, hence, supported the use of the simplified model following the principle of parsimony. The estimated β coefficients for the covariates were essentially the same as obtained before. Note that a model not including b_2 as explanatory variable but including the other covariates produced overall estimates $R_m^2 = 57.11\%$, $R_c^2 = 62.33\%$ and AIC = -170.28. The corresponding LRT (LR statistic = 42.87; $P < 0.001$) supported the inclusion of the VFA compositional balance b_2 in the model.

TABLE 5 (MODEL B RESULTS)

The relationships between CH_4 yield and the subset of VFA components involved in Eq. [4] are depicted in Fig. 3. Propionate was entered independently whereas acetate and butyrate concentrations, on the one hand, and the remaining VFA components, on the other hand, were entered together by geometric mean to obtain the [propionate, acetate · butyrate, others] subcomposition. The observed values were displayed on a ternary diagram, with the diet types distinguished by color and shape. Model B was used to produce expected Ln CH_4 yields from randomly generated samples of this subcomposition. An interpolated surface was fitted to them and used to fill the ternary diagram with colors according to the values.

FIGURE 3 (TERNARY DIAGRAM)

Each vertex of the ternary diagram corresponds with corner VFA subcompositions consisting of 100% of the component represented in there and 0% of any other. Maximum data variability occurred along the propionate to (acetate · butyrate) direction, with little variability observed in the direction pointing to the other vertex. The lowest expected CH_4 yields were associated with the greatest propionate concentrations, whereas the greatest expected CH_4 yields were associated with the greatest (acetate · butyrate) concentrations.

Using Model B as basis, separate LMMs were fitted to check whether the relationship between the disclosed VFA balance (Eq. [4]) and CH_4 yield was reproduced within a diet type. For both Concentrate and Mixed diet types we obtained a similar statistically highly significant positive estimated β coefficient for the balance representing the (acetate · butyrate) / propionate ratio ($P < 0.001$), thus supporting the association once the effects of DMI and ME were accounted for within these diet types (see summary tables in Appendix 2). As expected the amount of unexplained random variability increased in relation to the overall Model B. The effect of the VFA balance was however negligible within the Forage diet type ($\hat{\beta} = 0.073$; $P = 0.704$; summary table not shown). The number of animals on the Forage diet type was lower (45 animals versus 88 and 151 on the Concentrate and Mixed diet types respectively) and the forage constituents of the 2 trials which contributed to the Forage diet type were very different, which resulted in a sparse data set for this case.

DISCUSSION

The use of compositional methods within the natural sciences is rapidly increasing, with applications found in the study of environmental pollution (Howel, 2007), aroma volatile compounds (Korhonová et al., 2009), meat fatty acid composition (Ros-Freixedes and Estany, 2014) and free-ranging animal diets (Stewart et al., 2014), among others. It has been shown that treating the components in isolation, without relating them to each other, may lead to misleading or paradoxical conclusions. For example, the standard linear correlation measured between same 2 components can dramatically vary depending on the other components considered in the composition (Palarea-Albaladejo and Martín-Fernández, 2013). As an illustration of this, using our own VFA data, the correlation between iso-butyrate and iso-valerate is 0.62 when the entire 6-component VFA composition is considered, whereas it is -0.87 when working with only the [iso-butyrate, iso-valerate,

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valerate] subcomposition. Other common inconsistences include statistical confidence intervals covering nonsensical negative values or singularity and collinearity problems in linear models (Fox, 1997; Hron et al., 2012). In this work, we introduced compositional linear mixed models to investigate the association between rumen VFA and CH_4 yield from individual cattle, while accounting for the effects of other animal and diet-related covariates and the random variation originating from multiple experiments. Meaningful normalized log-contrasts (compositional balances) were defined between subsets of VFA components and their contribution to variability in CH_4 yields investigated.

Exploratory analysis revealed interesting proportionality associations between the VFA components and their connections with different diet types (Fig. 1). The VFA composition as a whole had a statistically significant association with CH_4 yield. The links between VFA components depicted in Fig. 2, and used in Model A1 (Table 4), described well the underlying stoichiometry of carbohydrate fermentation (Wolin, 1960). Thus, VFA associated with H_2 production, acetate and butyrate, were closely related and well separated from propionate and valerate which are associated with H_2 consumption. The branched chain VFA, iso-butyrate and iso-valerate were also closely related as might be expected given that these VFA are products of the catabolism of branched-chain AA. The closer alignment of the branched chain VFA with acetate and butyrate than with propionate and valerate is consistent with the requirement for these iso-acids by structural carbohydrate fermenting bacteria such as *Ruminococcus albus* (Allison and Bryant, 1963; Liu et al., 2014; Wang et al., 2015). Acetate, propionate, and butyrate are quantitatively the most important VFA in the rumen environment. When a log-contrast representing these 3 VFA (Eq. [4]) was used in Model A2 (Table 4), it was the only compositional balance having a statistically significant effect on CH_4 yield. Note that it is analogous to the so-called glucogenic ratio [(acetate + butyrate) / propionate]; the β coefficient was positive as would be expected because H_2 available for

CH_4 formation would be expected to be positively correlated with this ratio. Indeed, this is the best empirical representation of the mechanistic relationships proposed by Wolin (1960) relating CH_4 production to VFA. The relationship was consistent across concentrate and mixed diet types. Thus, compositional analysis produced relationships between VFA which described accurately the underlying biology. Note that using an alternative SBP to isolate a balance between butyrate only and propionate (in the numerator and denominator respectively of Eq. [1]) provided results very similar to those using the balance in Eq. [4]. We also implemented a SBP to test the compositional balance representing the exchange between acetate and propionate only, which has been advocated in Jansssen (2010) and Sauvant et al. (2011). Its effect on CH_4 yield was highly statistically significant as well ($P = 0.005$), however in this case it was not the only statistically significant balance in the model and, hence, the results were not so neat. This would then confer butyrate concentrations relative to propionate a leading role in the balance given by Eq. [4].

In developing models, other animal (DMI, BW) and nutritional (diet type, ME) covariates were tested and DMI, diet type, and ME were included and their influence on the model was in the direction expected. Thus, DMI was associated with a negative β coefficient recognizing that increased DMI is associated with increased rumen outflow and decreased extent of fermentation and therefore CH_4 production. Diet type was included as the diets fed were grouped into three distinct forage to concentrate ratios. The use of starch and NDF concentrations and the log-ratio NDF/starch were tested directly as covariates. However, they were highly related to each other and the information about the variation in chemical composition of diets was robustly represented by ME and diet type. From a statistical point of view, using diet type also provided greater consistency and numerical stability of the estimation process. Diet type was associated with a positive β coefficient as it changed from the Concentrate to the Mixed and then to the Forage diet type as expected from the mean

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values for CH_4 yield for the Concentrate (14.7 g/kg DMI) and Mixed (22.2 g/kg DMI) diet types (derived from Table 2). At first sight the positive β coefficient for the Forage diet type appears anomalous as mean CH_4 yield (17.6 g/kg DMI) was less than for the Mixed diet type. High forage diets are normally associated with high CH_4 yields because the high structural fiber content of these diets produces an acetate-dominated fermentation which was indeed observed (Table 3). The explanation for this apparent anomaly is that 2 diets in the Forage diet type had low ME concentrations (Table 1). Thus, the positive β coefficient associated with ME in the model likely adjusts responses for the digestibilities of these diets.

Many empirical models relating animal and diet variables to CH_4 have been produced (recent examples include Hristov et al., 2013a; Storlien et al., 2014; Ricci et al., 2013; Ramin and Huhtanen, 2013). Where CH_4 production is scaled either as g/kg DMI (as in the present study) or as kJ/MJ GE intake (Hristov et al., 2013a; Ramin and Huhtanen, 2013), terms related to DMI, diet digestibility and diet composition have been included in models. The inclusion of VFA in empirical models has been less common, largely because of scarcity of data. In some models diet composition has been used. Alemu et al., (2011) used VFA molar proportions predicted from the stoichiometry of fermentation to explain CH_4 and found that goodness of fit was model dependent. Ramin and Huhtanen (2013) compared the goodness of fit for CH_4 from models which including observed VFA proportions and concluded as here that models which included terms relating to combinations of VFA (acetate, propionate, and butyrate) gave superior explanations of CH_4 yield than individual VFA. However, all models noted above did not consider the compositional aspect of the data, that is, their natural relative and symmetric scale, and were based on treatment means for diets and did not use individual animal data in their analysis. Indeed, use of individual animal data in analysis of CH_4 production is not common. Mills et al. (2001) compared observed CH_4 production with that predicted from the mechanistic models of Dijkstra et al. (1992) and found relationships were superior at the treatment than individual animal level; however, data for VFA proportions was not reported. Robinson et al. (2010) generated a range of VFA concentrations by varying DMI of sheep ($n = 10$) fed a single diet of lucerne chaff and found that VFA concentrations only accounted for 25 – 30% of the variance. The present study was not comparable with Robinson et al. (2010) because of the narrow range of VFA molar proportions (700 – 730 mmol/mol acetate) used in that study. In the current study, a much greater variation in CH_4 yields and range of VFA proportions was available.

In reviewing mitigation options for reducing enteric CH_4 emissions, Hristov et al. (2013b) classified strategies into manure management practices and animal husbandry (which included genetics). Hristov et al. (2013b) noted that from genetic variation a reduction in predicted CH_4 production in the order of 11 to 26 percent was theoretically possible and that genomic selection tools could further increase the reduction in CH_4 production. However, effective application of genomic selection required significant international effort and collaboration to bring together relevant data because of the large datasets required. Because phenotypic CH_4 measurements are produced mainly using indirect respiration chambers, genetic progress is limited by slow throughput and cost. Therefore, there is a need for indirect proxy measurements for CH_4 emissions which are capable of rapid throughput and lower cost than chambers and these have been recently reviewed by Negussie et al. (2017) including critical appraisal of their limitations. The rumen samples which were used for measurement of VFA in the current study were of necessity single spot samples and, therefore, would not have captured changes in response to feed intake and fermentation. However, as cattle had not been given access to fresh feed for 25 h when sampled, variation due to short term feed intake would have been minimized and variability in VFA molar proportions between animals and experiments due to differences in feeding pattern reduced. Further cattle are

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often fasted before slaughter and therefore results from the current study could potentially apply to samples taken at slaughter.

Although we found the association between VFA composition and CH_4 yield highly statistically significant ($P < 0.001$), particularly through the (acetate · butyrate) / propionate compositional balance, there were other terms involved and the dataset included only beef cattle. If the use of VFA were implemented in practice, standardized protocols, such as those for determining residual feed intake (Basarub et al., 2003) may be appropriate. Other factors including rumen pH, protozoal population, and substrate utilization by methanogens, related to rumen fermentation of individual animals not captured by the VFA balances are probably responsible for unexplained variation and have been reviewed by Ellis et al. (2008). More recently, the genetic background of the animal has been shown to be important (King et al., 2011; Hernandez-Sanabria et al., 2013) as are inter-related phenotypic factors such as rumen size (Goopy et al., 2013), feed intake pattern (Carberry et al., 2014) and colonization of the rumen after birth (Yanez-Ruiz et al., 2015) which determine the host-specificity of the rumen microbiome (Weimer et al., 2010; Wallace et al., 2015). Indeed, in one of the experiments which contributed to the present data set (Rooke et al., 2016) the VFA pattern present in individual animals before imposition of experimental treatments was a significant covariate for subsequent samples. Apart from animal factors, differences in diet characteristics may influence the fate of H_2 in the rumen and variability in VFA pattern. Where nitrate was used as a mitigation strategy (e.g. Troy et al., 2015) reduction of nitrate to ammonia diverts H_2 away from VFA formation and increases the ratio of acetate to propionate. This is contradictory to balance b_2 (Table 5) and may have contributed to the unexplained variation in the overall model.

CONCLUSIONS

This work demonstrated the use and benefits of a novel statistical approach to the analysis of VFA compositions from individual animals and multiple experiments. The results were coherent with biological knowledge and emphasized the contribution of rumen VFA to explain cattle CH_4 yield at an individual animal level. Further research is needed to determine other possible contributing factors and investigate the scope for setting up more specialized empirical models within the same compositional framework to improve predictive capacity based on VFA measurements.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest in relation to this work.

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APPENDIX 1: SUMMARY STATISTICS OF THE VFA MOLAR PROPORTIONS AND THE COMPOSITIONAL BALANCE BASED ON THE (ACETATE · BUTYRATE) / PROPIONATE RATIO (b_2 IN EQ. [4], MODELS A2 AND B) PER STUDY AND DIET

Study[1,3]	Diet		Acetate	Propionate	Butyrate	Iso-butyrate	Iso-valerate	Valerate	b_2
Concentrate diet-type (forage less than 100 g/kg DM)									
1	1	Minimum	477	163	64	8	6	12	-0.71
		Q1[2]	554	240	77	10	10	14	-0.38
		Median	560	310	96	11	13	15	-0.24
		Q3[2]	566	329	122	14	23	17	0.10
		Maximum	645	417	155	17	33	27	0.54
2	3	Minimum	482	152	45	9	4	12	-0.78
		Q1	544	235	80	11	10	14	-0.36
		Median	562	284	95	12	16	16	-0.13
		Q3	590	318	131	16	28	20	0.03
		Maximum	668	411	221	20	72	31	0.47
4	7	Minimum	401	183	46	7	7	8	-0.93
		Q1	500	264	65	8	10	17	-0.57
		Median	535	335	80	14	15	18	-0.46
		Q3	561	402	123	17	21	22	-0.03
		Maximum	635	453	132	21	23	30	0.37
4	8	Minimum	527	126	59	9	10	6	-0.55
		Q1	561	181	66	11	12	10	-0.43
		Median	596	274	83	12	16	16	-0.21
		Q3	629	327	123	13	20	21	0.42
		Maximum	694	359	192	29	28	23	0.84
4	9	Minimum	495	177	50	10	10	10	-0.76
		Q1	527	239	65	15	16	16	-0.62
		Median	539	312	83	23	23	18	-0.30

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		Q3	586	364	106	23	26	25	0.05
		Maximum	627	415	176	26	42	28	0.45
Mixed diet-type (400 – 600 g forage / kg DM)									
1	2	Minimum	541	105	107	10	11	10	0.16
		Q1	650	128	111	11	13	12	0.28
		Median	665	170	121	12	14	13	0.46
		Q3	705	190	144	12	15	13	0.64
		Maximum	725	229	152	32	36	17	0.92
2	4	Minimum	596	90	76	11	12	10	-0.18
		Q1	634	158	103	13	16	13	0.17
		Median	648	174	125	15	18	14	0.41
		Q3	665	207	141	16	21	15	0.54
		Maximum	707	269	177	21	27	17	1.07
4	10	Minimum	548	137	75	9	11	8	-0.42
		Q1	614	170	85	13	12	12	0.03
		Median	653	196	110	14	16	16	0.20
		Q3	661	235	119	16	17	20	0.46
		Maximum	686	341	161	17	19	26	0.60
4	11	Minimum	624	118	82	10	6	11	0.03
		Q1	659	144	116	12	8	12	0.33
		Median	668	164	119	13	12	16	0.45
		Q3	682	182	140	14	14	18	0.65
		Maximum	707	229	164	16	15	22	0.79
4	12	Minimum	603	136	84	10	12	6	-0.01
		Q1	623	160	99	12	14	13	0.08
		Median	635	206	107	14	16	16	0.23
		Q3	670	231	113	16	18	18	0.40
		Maximum	717	248	186	19	32	23	0.70
6	14	Minimum	597	120	82	4	8	8	-0.06
		Q1	645	146	115	8	13	11	0.26

		Median	665	180	123	10	15	12	0.38
		Q3	683	200	136	11	16	13	0.63
		Maximum	723	244	154	15	20	24	0.75
6	15	Minimum	650	118	104	7	4	7	0.34
		Q1	664	134	113	8	11	12	0.42
		Median	690	154	122	10	14	12	0.50
		Q3	697	170	138	10	17	14	0.68
		Maximum	720	189	163	11	23	17	0.84
6	16	Minimum	623	140	97	6	10	9	0.23
		Q1	662	150	107	7	12	10	0.38
		Median	678	158	124	9	14	11	0.48
		Q3	695	174	141	10	17	14	0.55
		Maximum	715	197	161	11	21	21	0.65
6	17	Minimum	675	121	93	6	8	1	0.36
		Q1	684	144	113	8	13	9	0.48
		Median	690	147	123	8	13	11	0.56
		Q3	708	154	134	9	15	12	0.59
		Maximum	736	177	143	12	17	14	0.79
Forage diet-type (>700 g forage / kg DM)									
3	5	Minimum	726	106	53	4	5	4	0.04
		Q1	751	129	60	6	6	5	0.23
		Median	767	144	65	6	7	6	0.35
		Q3	792	165	70	8	8	6	0.46
		Maximum	804	189	78	11	14	8	0.69
3	6	Minimum	694	125	63	2	5	4	0.19
		Q1	718	155	69	6	7	5	0.29
		Median	734	163	82	7	8	6	0.31
		Q3	748	170	86	8	9	7	0.37
		Maximum	790	196	99	10	11	11	0.51
5	13	Minimum	662	114	72	0	9	6	0.13

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Q1	669	186	84	10	10	10	0.18
Median	676	202	91	10	11	10	0.19
Q3	698	203	98	12	12	11	0.22
Maximum	750	209	110	17	15	12	0.72

[1]1, Rooke et al., 2014; 2, Duthie et al., 2017; 3, Duthie et al., 2015; 4, Troy et al., 2015; 5, unpublished observations; 6, Troy et al., 2016.
[2]Q1, Q3; respectively first and third quartiles.
[3]To obtain individual data please contact richard.dewhurst@sruc.ac.uk.

APPENDIX 2: ESTIMATES FOR THE CONCENTRATE AND MIXED DIET TYPES OF THE SIMPLIFIED MIXED MODEL FOR METHANE EMISSIONS (G/KG DMI IN LOG SCALE) USING THE COMPOSITIONAL BALANCE BASED ON THE (ACETATE · BUTYRATE) / PROPIONATE RATIO (b_2 IN EQ. [4], MODELS A2 AND B) AND THE STATISTICALLY SIGNIFICANT COVARIATES FROM THE OVERALL MODEL B

Concentrate diet type				
Fixed effects	$\hat{\beta}$	SE	t-value	P-value
Intercept	0.744	2.106	0.35	0.725
b_2	0.297	0.049	6.03	< 0.001
Ln ME, MJ/kg DM	1.026	0.834	1.23	0.222
Ln DMI, g/kg BW$^{0.75}$/d	-0.279	0.098	-2.85	0.006
$R_m^2 = 32.33\%$[1], $R_c^2 = 38.69\%$[1], $\hat{\sigma}_f = 0.06$[2], $\hat{\sigma} = 0.17$[2]				

Mixed diet type				
Fixed effects	$\hat{\beta}$	SE	t-value	P-value
Intercept	-0.805	1.520	-0.53	0.597
b_2	0.206	0.050	4.10	<0.001
Ln ME, MJ/kg DM	1.806	0.614	2.94	0.004
Ln DMI, g/kg BW$^{0.75}$/d	-0.289	0.060	-4.82	< 0.001
$R_m^2 = 21.46\%$, $R_c^2 = 41.53\%$, $\hat{\sigma}_f = 0.07$, $\hat{\sigma} = 0.13$				

[1]R_m^2, R_c^2; respectively % variance explained by fixed terms (marginal) and by both fixed and random terms (conditional).
[2]$\hat{\sigma}_f$, $\hat{\sigma}$; respectively estimated standard deviations of the random effects and random error terms.

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Table 1. Sources of data and nutritional characteristics of the diets used in the study

Study[1]	Diet	Forage, g/kg DM	Starch, g/kg DM	NDF, g/kg DM	ME[2], MJ/kg DM
Concentrate diet type (forage less than 100 g/kg DM)					
1	1	80	412	254	12.3
2	3	79	415	248	12.8
4	7	84	439	227	12.2
4	8	80	476	204	12.0
4	9	78	416	211	12.9
Mixed diet type (400 – 600 g forage / kg DM)					
1	2	484	234	388	11.9
2	4	505	284	374	12.0
4	10	490	298	289	11.6
4	11	499	318	272	11.4
4	12	497	262	280	12.2
6	14	557	281	308	11.6
6	15	558	308	295	11.4
6	16	555	264	317	11.9
6	17	556	247	313	11.6
Forage diet type (>700 g forage / kg DM)					
3	5	774	65	771	7.4
3	6	1000	0	693	8.1
5	13	1000	36	473	10.7

[1]1, Rooke et al., 2014; 2, Duthie et al., 2017; 3, Duthie et al., 2015; 4, Troy et al., 2015; 5, unpublished observations; 6, Troy et al., 2016a.
[2]ME estimated from feed composition (Rymer and Agnew, 2004).

Table 2. Methane emissions, DMI, and BW of cattle included in database

Study[1]	Diet	*n*	BW, kg		DMI, kg/d		Methane, g/d		Methane, g/kg DMI	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Concentrate diet type (forage less than 100 g/kg DM)										
1	1	15	667	45.1	11.2	2.12	153	44.6	13.9	4.07
2	3	35	635	55.4	10.6	1.74	147	31.2	13.9	2.32
4	7	12	675	41.0	10.0	1.55	148	48.1	14.6	3.31
4	8	13	675	54.3	9.1	2.09	136	27.4	15.3	3.39
4	9	13	687	55.7	9.7	2.04	149	32.4	15.8	3.58
Mixed diet type (400 – 600 g forage / kg DM)										
1	2	13	652	37.4	9.4	1.09	218	46.6	23.4	4.99
2	4	33	605	54.5	9.2	1.59	189	40.2	20.5	3.05
4	10	12	703	40.6	9.8	1.55	235	36.8	24.3	5.06
4	11	12	707	30.2	10.4	1.56	212	25.4	20.6	2.60
4	12	12	705	25.7	10.5	1.53	242	35.2	23.2	2.10
6	14	17	673	26.1	10.4	1.80	245	46.9	23.8	3.58
6	15	16	649	52.5	9.8	2.18	214	42.8	22.1	2.68
6	16	18	652	24.1	10.2	1.41	238	38.6	23.4	2.79
6	17	18	652	33.9	10.2	1.86	210	27.5	20.9	2.48
Forage diet type (>700 g forage / kg DM)										
3	5	16	639	78.5	9.4	1.84	126	24.0	13.8	3.36
3	6	17	607	97.1	10.1	1.65	160	33.3	16.5	5.55
5	13	12	704	58.3	13.7	1.77	308	36.9	22.6	2.12

[1]1, Rooke et al., 2014; 2, Duthie et al., 2017; 3, Duthie et al., 2015; 4, Troy et al., 2015; 5, unpublished observations; 6, Troy et al., 2016.

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Table 3. Compositional geometric mean (CGM), overall and by diet group, and relative variation matrix for the VFA composition across experiments

		Acetate	Propionate	Butyrate	Iso-butyrate	Iso-valerate	Valerate
CGM[1], mmol/mol	Overall	656	199	106	11	14	13
	Concentrate	574	282	96	14	17	17
	Mixed	670	170	121	11	15	13
	Forage	739	162	77	8	8	7
Variation matrix[2]	Acetate		0.19	0.10	0.18	0.25	0.28
	Propionate	0.19		0.29	0.18	0.29	0.16
	Butyrate	0.10	0.29		0.18	0.21	0.23
	Iso-butyrate	0.18	0.18	0.18		0.13	0.15
	Iso-valerate	0.25	0.29	0.21	0.13		0.22
	Valerate	0.28	0.16	0.23	0.15	0.22	

[1]Normalized vector of geometric means of the VFA composition.
[2]Matrix of log-ratio variances between pairs of VFA components.

Table 4. Sequential binary partitions (SBP) providing alternative balance coordinate representations ($b_i, i = 1, \ldots, 5$) of the VFA composition and estimates of the associated compositional mixed model for methane emissions (g/kg DMI) in log scale, Models A1 (top) and A2 (bottom)

	SBP[1]	b_1	b_2	b_3	b_4	b_5	Fixed effects	$\hat{\beta}$	SE	t-value	P-value
	Iso-valerate	+	+		+		Intercept	-2.134	1.041	-2.05	0.041
	Iso-butyrate	+	+		–		b_1	0.163	0.035	4.66	<0.001
	Butyrate	+	–			+	b_2	-0.122	0.043	-2.82	0.005
	Acetate	+	–			–	b_3	0.158	0.047	3.36	0.001
Model A1	Valerate	–		+			b_4	0.043	0.044	0.96	0.338
	Propionate	–		–			b_5	-0.017	0.078	-0.21	0.832
	r	4	2	1	1	1	Mixed diet	0.374	0.034	11.13	<0.001
	s	2	2	1	1	1	Forage diet	0.732	0.101	7.23	0.002
							Ln ME, MJ/kg DM	1.753	0.250	7.01	<0.001
$R_m^2 = 64.65\%$[2] AIC = -207.51[3]							Ln BW, kg	0.195	0.128	1.52	0.129
$R_c^2 = 68.60\%$[2] $\hat{\sigma}_f = 0.06$[4] $\hat{\sigma} = 0.16$[4]							Ln DMI, g/kg BW$^{0.75}$/d	-0.400	0.059	-6.84	0.000
	Acetate	+	+	+			Intercept	-2.134	1.041	-2.05	0.041
	Butyrate	+	+	–			b_1	0.008	0.039	0.22	0.829
	Propionate	+	–				b_2	0.256	0.050	5.11	<0.001
	Valerate	–			+		b_3	0.017	0.078	0.21	0.832
Model A2	Iso-butyrate	–			–	+	b_4	0.026	0.036	0.72	0.470
	Iso-valerate	–			–	–	b_5	-0.043	0.045	-0.96	0.338
	r	3	2	1	1	1	Mixed diet	0.374	0.034	11.13	<0.001
	s	3	1	1	2	1	Forage diet	0.732	0.101	7.23	0.002
							Ln ME, MJ/kg DM	1.753	0.250	7.01	<0.001
$R_m^2 = 64.65\%$ AIC = -207.51							Ln BW, kg	0.195	0.128	1.52	0.129
$R_c^2 = 68.60\%$ $\hat{\sigma}_f = 0.06$ $\hat{\sigma} = 0.16$							Ln DMI, g/kg BW$^{0.75}$/d	-0.400	0.059	-6.84	<0.001

[1] Symbol + means that a VFA component is allocated to the numerator of the corresponding compositional balance b_i, whereas – means that it is allocated to the denominator. Letters r and s refer to the number of VFA components in numerator and denominator respectively.
[2] R_m^2, R_c^2; respectively % variance explained by fixed terms (marginal) and by both fixed and random terms (conditional).
[3] Akaike information criterion measure of the relative quality of the model for the full data set.
[4] $\hat{\sigma}_f$, $\hat{\sigma}$; respectively estimated standard deviations of the random effects and random error terms.

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Table 5. Estimates of the simplified mixed model (Model B) for methane emissions (g/kg DMI in log scale) using the compositional balance based on the (acetate · butyrate) / propionate ratio (b_2 in Eq. [4], Model A2) and the statistically significant covariates

Fixed effects	$\hat{\beta}$	SE	t-value	P-value
Intercept	-0.837	0.613	-1.36	0.174
b_2	0.243	0.035	6.97	< 0.001
Mixed diet	0.374	0.033	11.47	< 0.001
Forage diet	0.720	0.105	6.83	0.002
Ln ME, MJ/kg DM	1.725	0.246	6.99	< 0.001
Ln DMI, g/kg $BW^{0.75}$/d	-0.356	0.051	-6.96	< 0.001

$R_m^2 = 63.51\%$[1] AIC = -211.15[2]

$R_c^2 = 68.72\%$[1] $\hat{\sigma}_f = 0.06$[3] $\hat{\sigma} = 0.16$[3]

[1]R_m^2, R_c^2; respectively % variance explained by fixed terms (marginal) and by both fixed and random terms (conditional).
[2]Akaike information criterion measure of the relative quality of the model for the full data set.
[3]$\hat{\sigma}_f$, $\hat{\sigma}$; respectively estimated standard deviations of the random effects and random error terms.

Figure 1. Compositional biplot of the VFA (mmol/mol) data set with the components represented by rays and the collected samples represented by points according to concentrate, mixed, and forage diet types. The links (dashed lines) between arrowheads are proportional to the log-ratio variances between the corresponding VFA components.

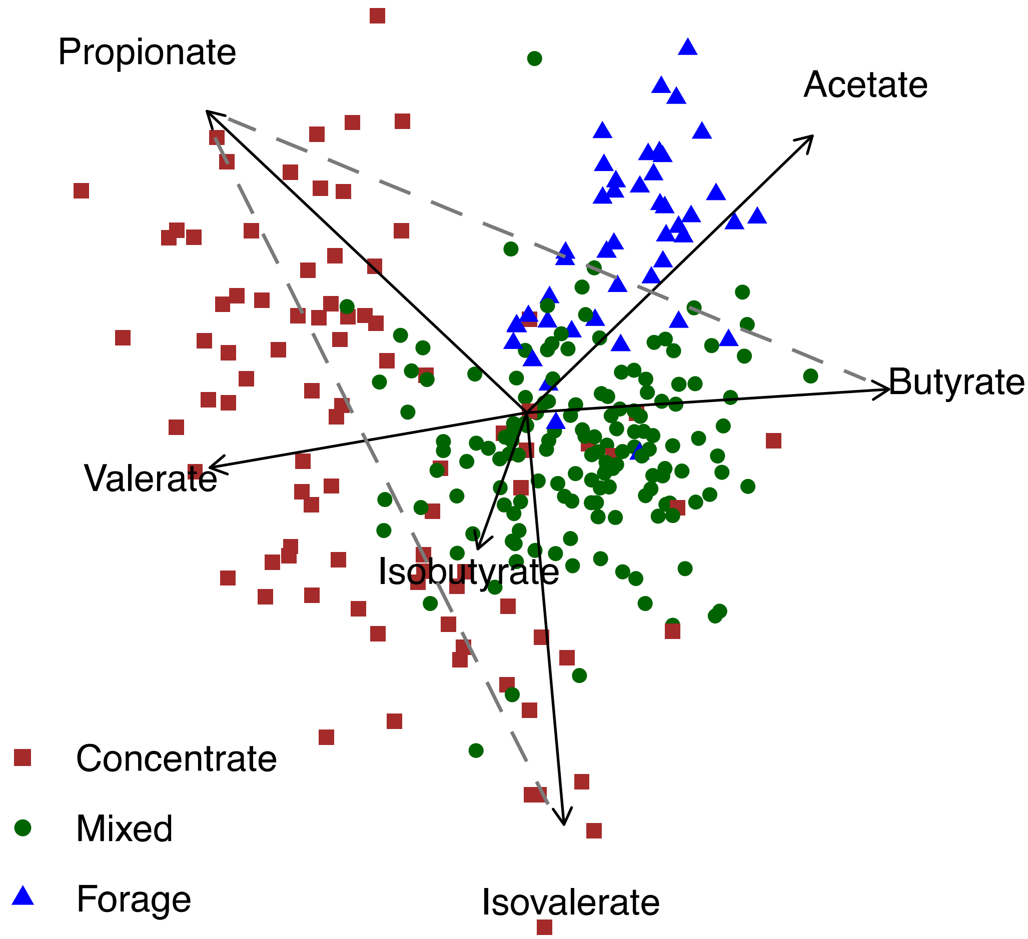

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Figure 2. Groupings of VFA (mmol/mol) components according to proportionality relationships from the variation matrix and associated balances ($b_i, i = 1, \ldots, 5$) for Model A1.

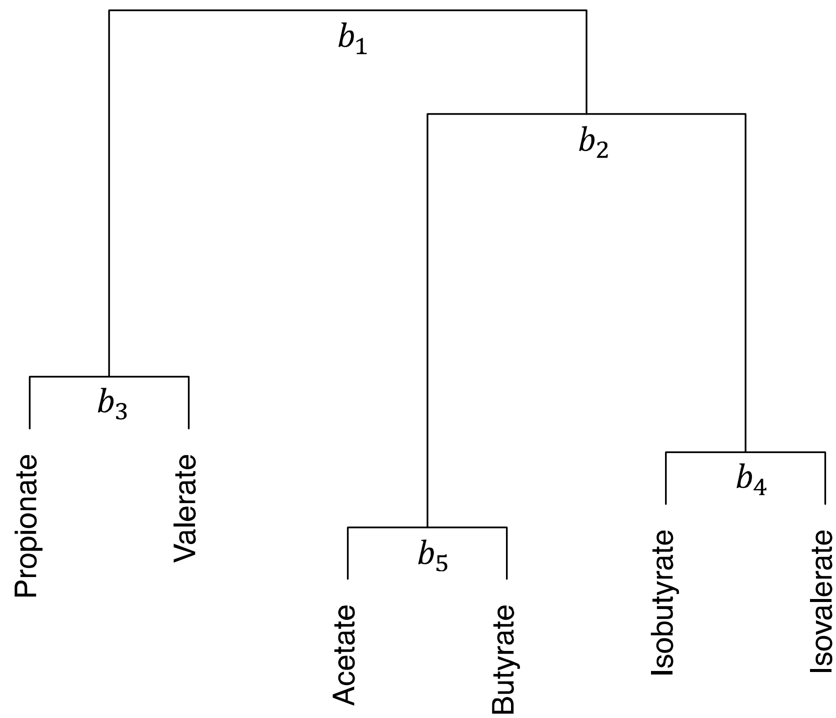
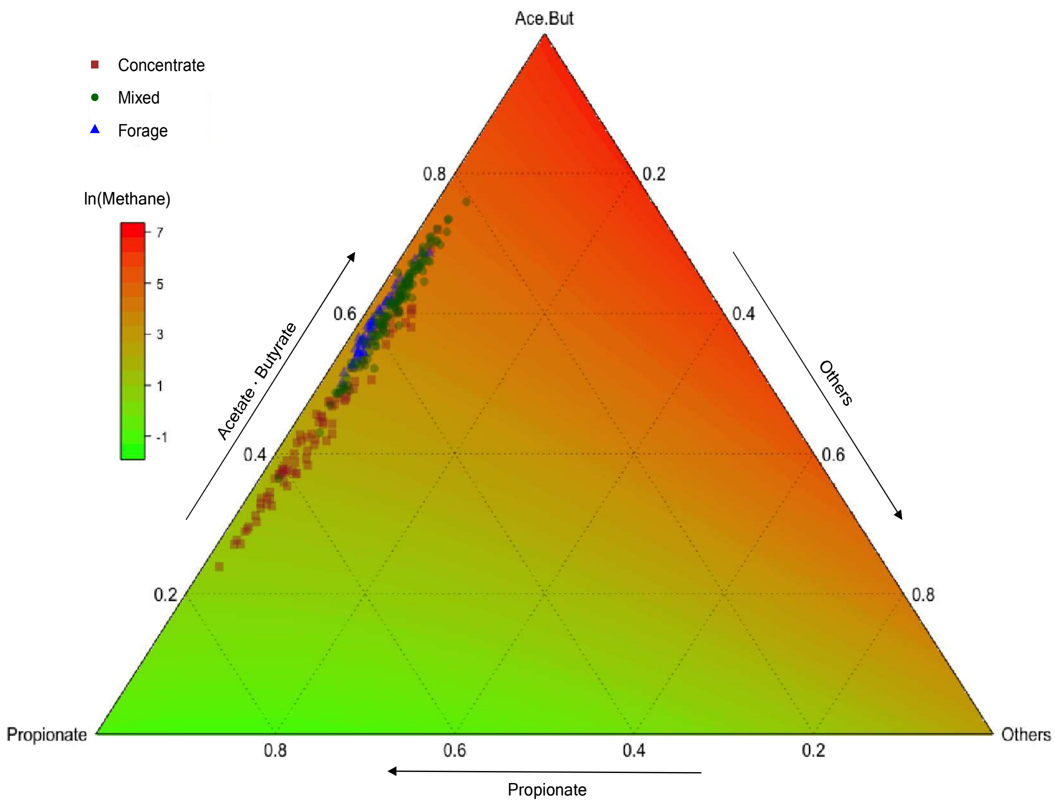
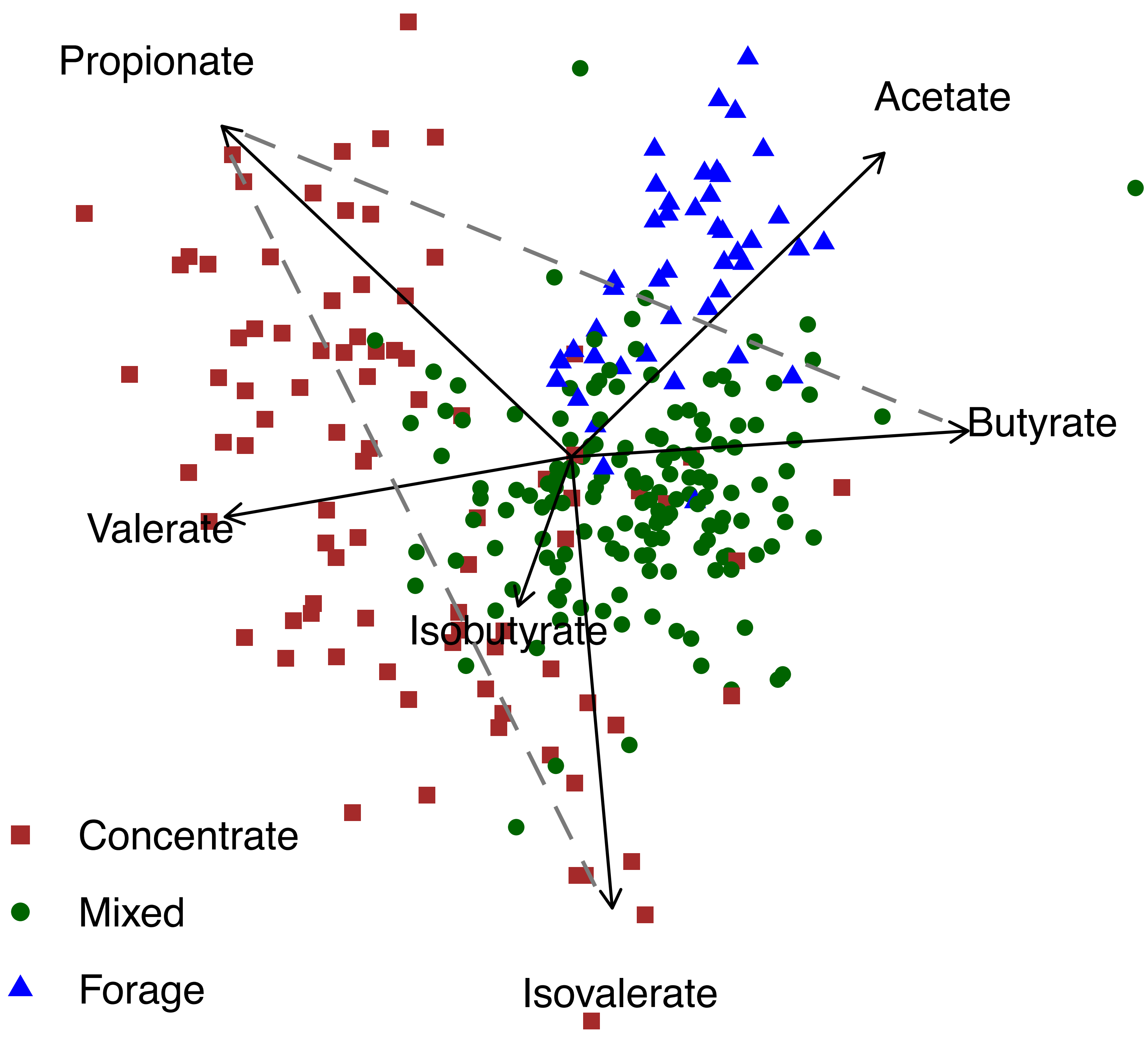
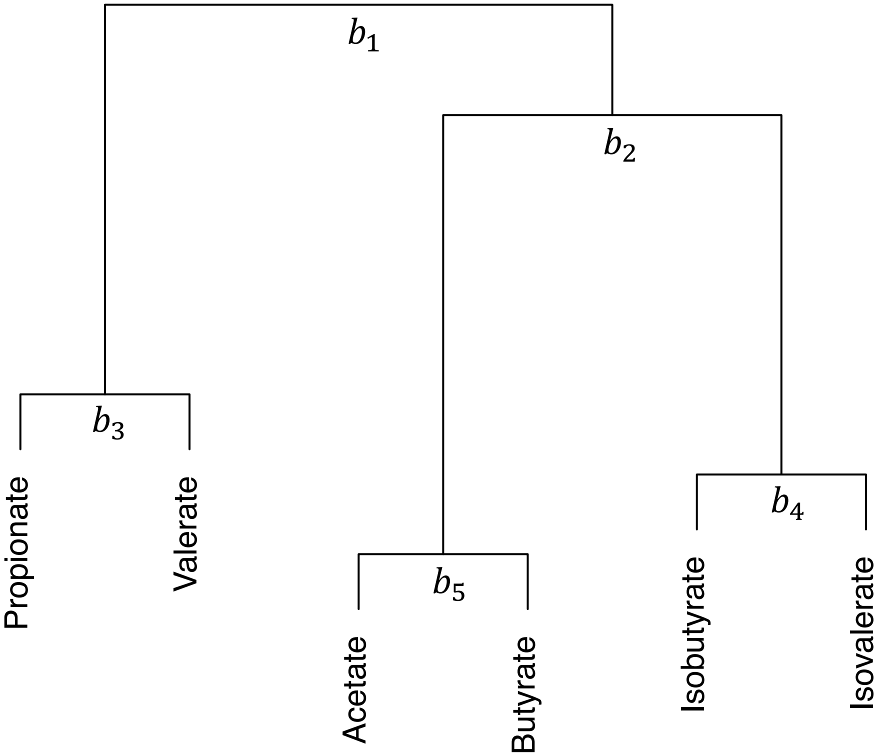
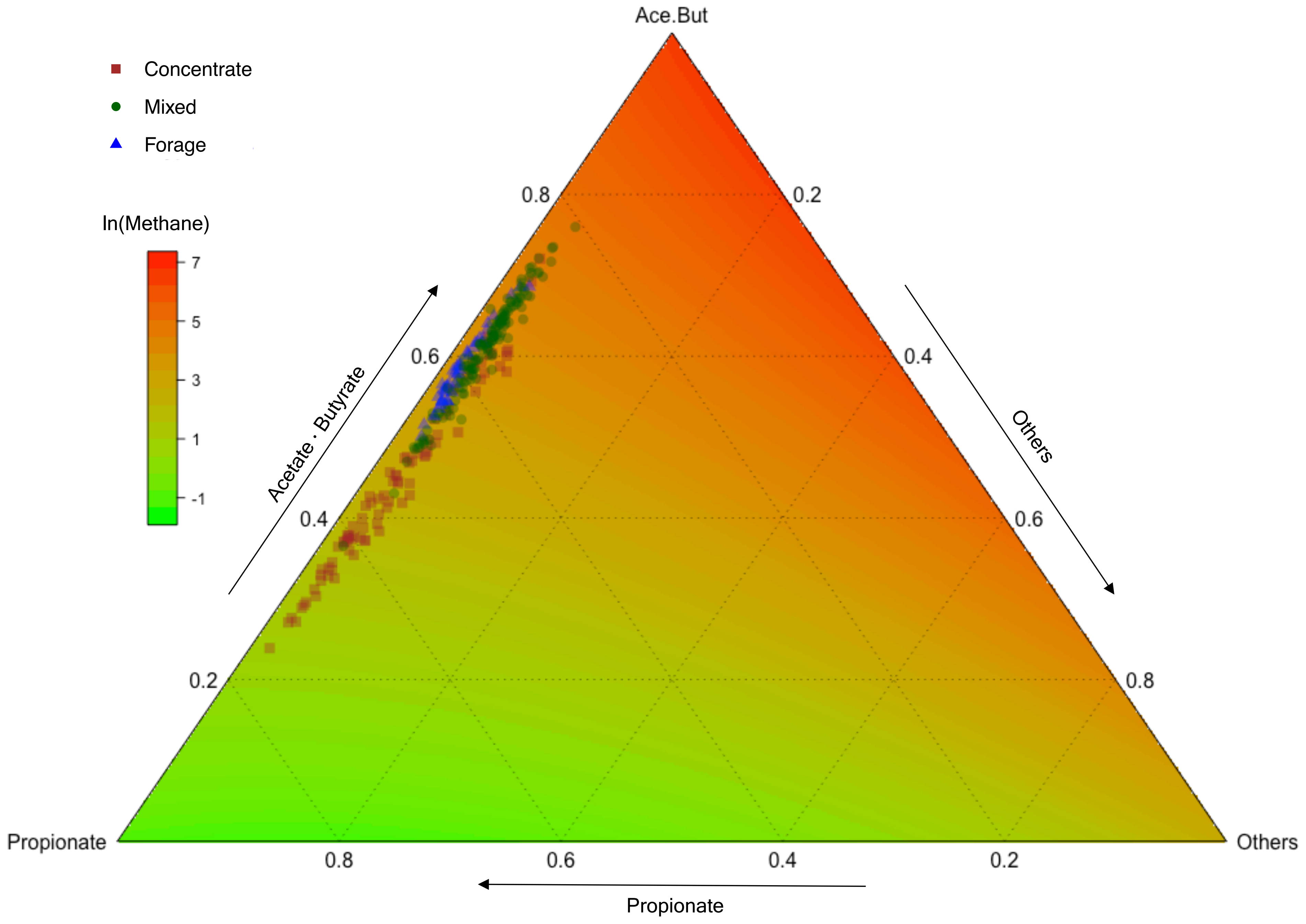


Figure 3. Ternary diagram of the [acetate · butyrate (Ace.But), propionate, others] VFA subcomposition for concentrate, mixed, and forage diet types and expected methane emissions (g/kg DMI in log scale) from Model B.




Propionate
Acetate
Butyrate
Valerate
Isobutyrate
Isovalerate
Concentrate
Mixed
Forage


b_1
b_2
b_3
b_4
b_5
Propionate
Valerate
Acetate
Butyrate
Isobutyrate
Isovalerate



Ace.But
Concentrate
Mixed
Forage
ln(Methane)
7
5
3
1
-1
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
Acetate · Butyrate
Others
Propionate
Others
0.8
0.6
0.4
0.2
Propionate